# A Mass Spectrometry Assay to Simultaneously Analyze ROS1 and RET Fusion Gene Expression in Non–Small-Cell Lung Cancer

*Priyanga Wijesinghe, PhD, Gerold Bepler, MD, PhD, and Aliccia Bollig-Fischer, PhD*

**Introduction:** ROS1 and RET gene fusions were recently discovered in non–small-cell lung cancer (NSCLC) as potential therapeutic targets with small-molecule kinase inhibitors. The conventional screening methods of these fusions are time-consuming and require samples of high quality and quantity. Here, we describe a novel and efficient method by coupling the power of multiplexing polymerase chain reaction and the sensitivity of mass spectrometry.

**Methods:** The multiplex mass spectrometry platform simultaneously tests samples for the expression of nine ROS1 and six RET fusion genes. The assay incorporates detection of wild-type exon junctions immediately upstream and downstream of the fusion junction to exclude false-negative results. To flag false-positives, the system also comprises two independent assays for each fusion gene junction.

**Results:** The characteristic mass spectrometric peaks of the gene fusions were obtained using engineered plasmid constructs. Specific assays targeting the wild-type gene exon junctions were validated using complimentary DNA from lung tissue of healthy individuals. The system was further validated using complimentary DNA derived from NSCLC cell lines that express endogenous fusion genes. The expressed ROS1-SLC34A2 and CCDC6-RET gene fusions from the NSCLC cell lines HCC78 and LC-2/ad, respectively, were accurately detected by the novel assay. The assay is extremely sensitive, capable of detecting an event in test specimens containing 0.5% positive tumors.

**Conclusion:** The novel multiplexed assay is robustly capable of detecting 15 different clinically relevant RET and ROS1 fusion variants. The benefits of this detection method include exceptionally low sample input, high cost efficiency, flexibility, and rapid turnover.

**Key Words:** Fusion gene, Non–small-cell lung cancer, Mass spectrometry, ROS1, RET.

(*J Thorac Oncol.* 2015;10: 381–386)

The application of high-throughput sequencing methods to investigate the genomic landscape of non–small-cell lung cancer (NSCLC) has led to the discovery of greater numbers of putative driver oncogene mutations in lung cancer.[1] Specifically, transcriptome sequencing has enabled the discovery of novel ROS1 and RET fusion genes resulting from somatic tumor genome rearrangements, and subsequent research has verified the oncogenic potential for some.[2–8] Although the prevalence of ROS1 and RET fusion genes are approximately 1% to 2% in tumors from an unselected population of NSCLC patients,[6,9] there is great interest to establish robust methods for detection with application to research and clinical outcomes. The expression of ROS1 and RET fusion genes occurs in the absence of other cancer driver mutations, and this mutual exclusivity can be used to enrich patient populations for treatment with targeted agents.[8] Also, NSCLC patients expressing ROS1 or RET fusion genes show unique clinicopathologic features that can facilitate therapeutic selection (e.g., relatively younger age, never-smoker with adenocarcinoma histology).[6,9] Finally, at the protein level, ROS1 and RET are kinases and small-molecule kinase inhibitor drugs shown to target ROS1 (e.g., crizotinib) and RET (e.g., cabzantinib, vandetanib, sorafenib, and sunitinib) in clinical trials.[9,10]

Fluorescent in situ hybridization , reverse transcriptase polymerase chain reaction (RT-PCR), and immunohistochemistry are among the widely used techniques currently applied in clinical settings for the detection of fusion genes,[6,7,9] and each has limitations.[11] Recently, Sakai et al [12] promoted a multiplexed approach that uses primer-targeted amplification and primer extension reactions coupled with matrix-assisted laser desorption ionization time-of-flight mass spectrometry as a robust method for diagnosis of tumors harboring *ALK*-activating rearrangements. The same research group was successful in detecting fusion genes when applying the method to the analysis of cell-free RNA in serum from lung cancer patients.[13] This is an attractive method because it requires very

Barbara Ann Karmanos Cancer Institute and Department of Oncology, Wayne State University School of Medicine, Detroit, MI.

Disclosure: The authors declare no conflict of interest.

This work was funded by Department of Defense contract number W81XWH-10-2-0068, which was awarded and administered by the US Army Medical Research & Materiel Command (USAMRMC) and the Telemedicine & Advanced Technology Research Center (TATRC), at Fort Detrick, MD. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The views, opinions, and findings contained in this publication are those of the authors and do not necessarily reflect the views of the Department of Defense and should not be construed as an official DoD/Army position, policy, or decision. Additional support comes from NIH Center grant P30 CA022453 to the Karmanos Cancer Institute at Wayne State University.

Address for correspondence: Aliccia Bollig-Fischer, Barbara Ann Karmanos Cancer Institute and Department of Oncology, Wayne State University School of Medicine, 4100 John R, Detroit, MI 48201. E-mail: bollig@karmanos.org.

DOI: 10.1097/JTO.0000000000000337



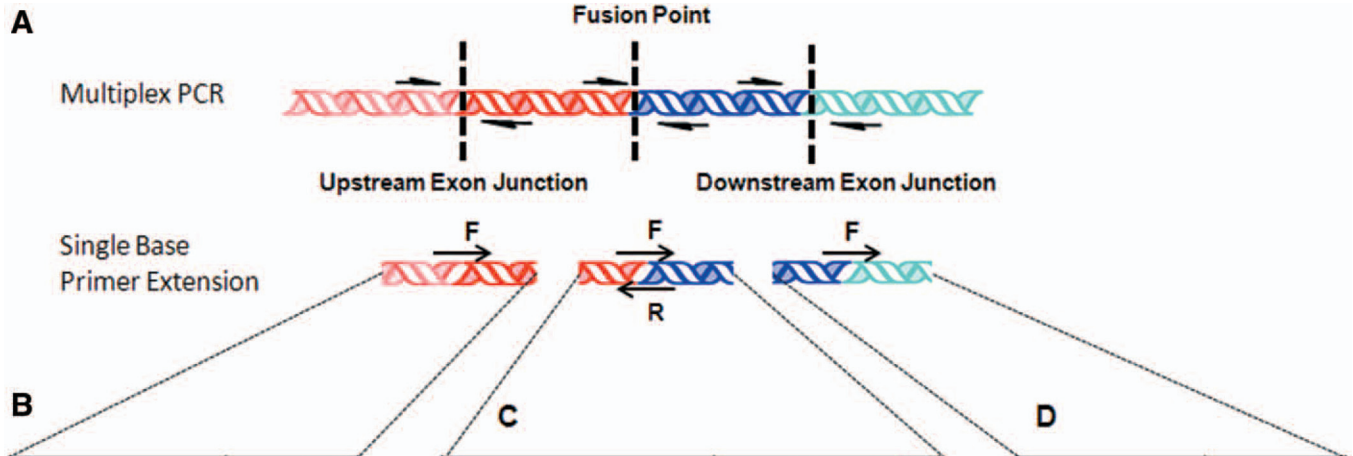
ISSN: 1556-0864/15/1002-0381

little specimen, it is cost-competitive and time-efficient compared with the other approaches, and it was previously demonstrated to be applicable to formalin-fixed paraffin-embedded specimens.[14] Based on these advantages, we elected to adapt the same test platform for detecting the expression of documented oncogenic ROS1 and RET fusion genes. Our novel multiplexed design strategy represents advancement over other mass spectrometry-based tests described in the literature in that it incorporates controls to indicate false-positive and false-negative results. Herein, we describe the multigene, multiplexed assay design and validation.

## MATERIALS AND METHODS

### MassARRAY Procedure Overview and Primer Design

The assay tests for the presence of fusion genes in specimen-derived mRNA converted to double-stranded complimentary DNA (cDNA). Once mRNA is converted, the procedure continues with a polymerase chain reaction (PCR) amplification step where a short sequence (on average 100 base pairs) surrounding the fusion gene junction or adjacent wild-type exon junction (incorporated as an integrated control to verify assay performance) is amplified. Next, an extension reaction is performed; this adds a single base to an extension primer spanning the putative fusion junction or adjacent wild-type exon junction. Finally, matrix-assisted laser desorption time-of-flight mass spectrometry analysis is done to detect the expected mass spectra of the extension primers. Detailed protocol of the assay design and data analysis is provided in the Supplemental Digital Content 1 (http://links.lww.com/JTO/A693). A total of 60 reactions—30 forward and reverse reactions at the fusion point and 30 reactions at upstream and downstream exon junctions—are multiplexed into 12 wells (Fig. 1).




B

| | Upstream Exon Junction | Extended mass (*m/z*) |
|---|---|---|
| 1. | *TPM3 7-8* | 5877.9 |
| 2. | *SDC4 1-2* | 5458.6 |
| 3. | *SDC4 3-4* | 5335.5 |
| 4. | *SLC34A2 3-4* | 5883.9 |
| 5. | *SLC34A2 12-13* | 5353.5 |
| 6. | *CD74 5-6* | 6732.4 |
| 7. | *CD74 5-6* | 6756.4 |
| 8. | *EZR 9-10* | 6159.0 |
| 9. | *LRIG3 15-16* | 6061.0 |
| 10. | *KIF5B 14-15* | 5514.5 |
| 11. | *KIF5B 15-16* | 5818.8 |
| 12. | *KIF5B 21-22* | 5572.7 |
| 13. | *KIF5B 22-23* | 6485.3 |
| 14. | *KIF5B 23-24* | 6108.0 |
| 15. | *CCDC6 (ATG-start)* | 5538.7 |

C

| | Fusion Gene | Extended mass (*m/z*) | |
|---|---|---|---|
| | | Forward | Reverse |
| 1. | *TPM3-ROS1 T8;R35* | 5553.5 | 5392.5 |
| 2. | *SDC4-ROS1 S2; R32* | 5633.5 | 5731.8 |
| 3. | *SDC4-ROS1 S4; R34* | 6186.9 | 5394.6 |
| 4. | *SLC34A2-ROS1 SL4; R32* | 5649.5 | 6036.0 |
| 5. | *SLC34A2-ROS1 SL13; R32* | 5931.7 | 5762.8 |
| 6. | *CD74-ROS1 C6; R32* | 5531.5 | 6098.0 |
| 7. | *CD74-ROS1 C6; R34* | 6461.1 | 7284.8 |
| 8. | *EZR-ROS1 E10; R34* | 5963.8 | 6900.5 |
| 9. | *LRIG3-ROS1 L16;R35* | 5512.6 | 5745.6 |
| 10. | *KIF5B-RET K15;R12* | 7126.7 | 5975.9 |
| 11. | *KIF5B-RET K16; R12* | 6643.4 | 6370.2 |
| 12. | *KIF5B-RET K22; R12* | 6494.3 | 5981.9 |
| 13. | *KIF5B-RET K23; R12* | 5499.6 | 5420.6 |
| 14. | *KIF5B-RET K24; R11* | 5441.6 | 5770.8 |
| 15. | *CCDC6-RET C1;R12* | 6349.2 | 5767.8 |

D

| | Downstream Exon Junction | Extended mass (*m/z*) |
|---|---|---|
| 1. | *ROS1 35-36* | 6363.0 |
| 2. | *ROS1 32-33* | 7656.0 |
| 3. | *ROS1 34-35* | 5530.5 |
| 4. | *ROS1 32-33* | 6704.3 |
| 5. | *ROS1 32-33* | 6391.1 |
| 6. | *ROS1 32-33* | 6391.1 |
| 7. | *ROS1 34-35* | 5819.7 |
| 8. | *ROS1 34-35* | 5530.5 |
| 9. | *ROS1 35-36* | 6554.3 |
| 10. | *RET 12-13* | 5479.6 |
| 11. | *RET 12-13* | 5479.6 |
| 12. | *RET 12-13* | 6138.0 |
| 13. | *RET 12-13* | 6138.0 |
| 14. | *RET 11-12* | 6140.0 |
| 15. | *RET 12-13* | 6427.2 |

**FIGURE 1.** Schematic depicting the major steps of the assay and primers designed to detect the fusion junctions and the adjacent wild-type exon boundary junctions. *A*, Cartoon generalizing multiplexed primer targets. *B*, List of upstream exon junction–targeted primers for the 15 fusion genes and their corresponding masses after the primer extension. *C*, List of fusion junction–targeted extension primers and the respective mass peaks from both forward and reverse directions. *D*, List of downstream exon junction–targeted primers and the respective mass peaks. *PCR*, polymerase chain reaction.

Target sequences of the fusion genes (Supplemental Digital Content 2, http://links.lww.com/JTO/A694) were obtained from published work[5,6] and cross-checked with the National Center for Biotechnology Information (NCBI) sequences (*Homo sapiens* nucleotide Basic Local Alignment Search Tool [BLAST]). The transcript ID of each gene involved in fusions was obtained from the Ensemble Genome Browser (http://useast.ensembl.org/index.html). The Exon Extraction Tool (Sequenom, San Diego, CA) was used to annotate exon junctions using the above transcript IDs. The immediate upstream and downstream exon junctions of the fusion point were annotated manually. Assay Designer 4.0 software (Sequenom, San Diego, CA) was used to determine the product sequences for PCR amplification and extension primers along with their predicted masses. PCR primers (100 μM, 25 nmol) and extension primers (200 μM, 100 nmol) were ordered from Integrated DNA Technologies (IDT).

## Assay Validation

Plasmids comprising synthetic fusion genes—exact cDNA surrogates for the endogenously expressed ROS1

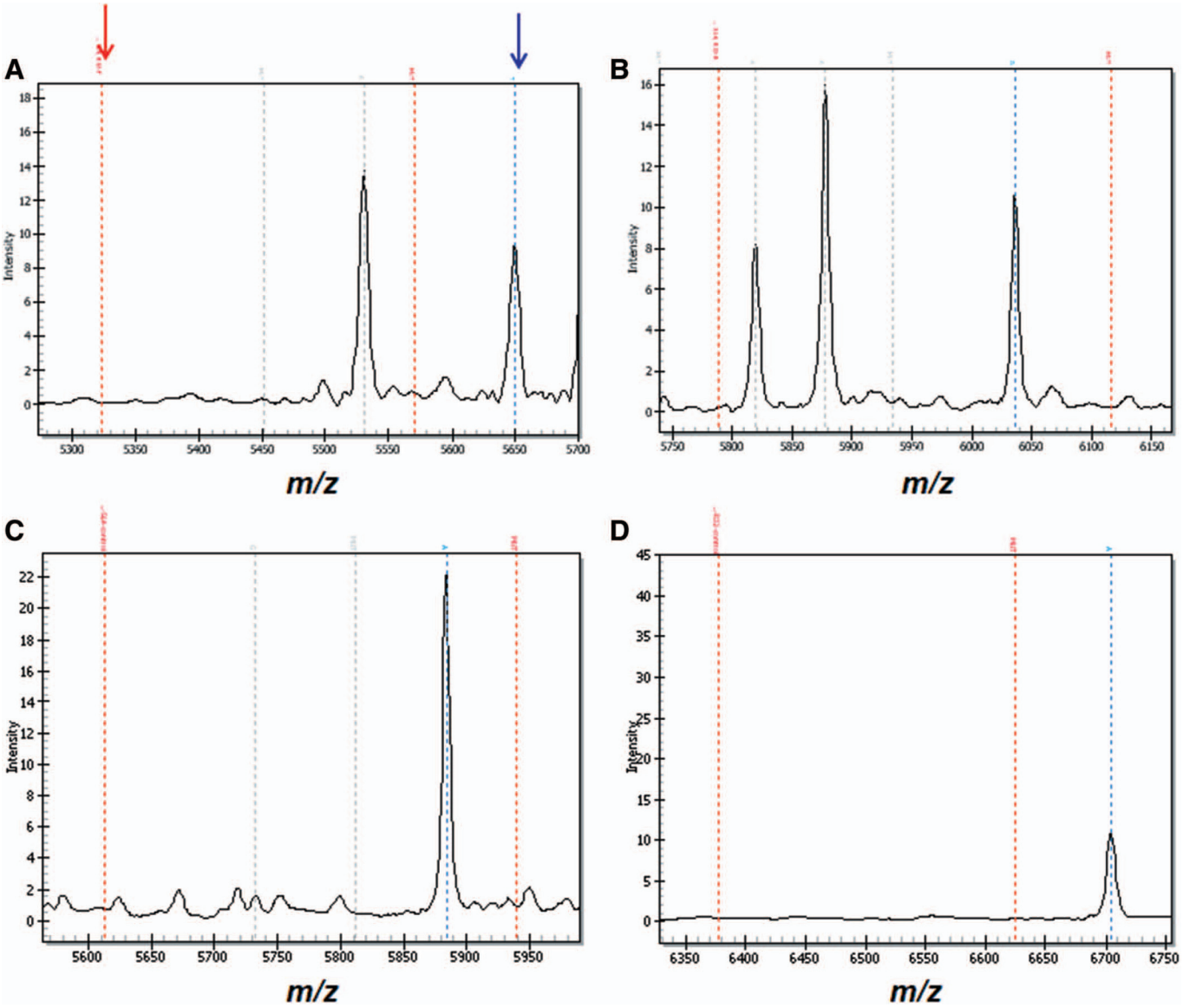



**FIGURE 2.** Detection of endogenous expression of the SLC34A2-ROS1 (SL4; R32) fusion gene in complimentary DNA derived from the HCC78 cell line. When the fusion gene is present, the characteristic mass spectrometry peak will appear in the analytical output of the MassARRAY system (blue arrow) along with the location of depleted extension primer (red arrow). If peaks from other reactions not directly related to the reaction lie in the same framed area of the spectrum, they are shown in gray (inactive mode). The results for the four reactions relevant to testing for this fusion gene are shown: (*A*) peak corresponding to the extension primer reaction targeting the fusion junction in the forward direction; (*B*) peak corresponding to the extension primer targeting the fusion junction in the reverse direction; (*C*) peak corresponding to the control extension primer targeting the upstream exon junction; (*D*) peak at predicted mass corresponding to control extension primer reaction targeting the downstream exon junction.

and RET fusion genes—were used to validate the specificity and sensitivity of each assay in multiplex fashion; they were acquired from DNA2.0 Inc. (Menlo Park, CA). The cDNA sequences of the 15 synthesized fusion genes are given in Supplemental Digital Content 3 (http://links.lww.com/JTO/A695). The copy number of the plasmid cDNA was determined based on the equation: 6.022E23 [mass of DNA (g)]/[plasmid size (base pair) × 660 g/mol)]. To validate the function of control amplification and extension primers targeting wild-type gene exon junctions, human lung cDNA (from healthy donors) was purchased from Clontech Laboratories, CA. To observe mass spectra for wild-type exon junctions, 0.075 ng/well of healthy cDNA was spiked with plasmid DNA. To test for endogenously occurring SLC34A2-ROS1 (SL4; R32) and the CCDC6-RET fusion genes, cDNA from HCC78 and LC-2/ad lung cancer–derived cell lines were purchased from Creative Bioarray (New York, NY). To check the sensitivity of the assay, the cancer cell cDNA was diluted with normal lung cDNA and tested according to ratios in Figure 4.

## RESULTS

Being that the occurrence of each of these fusion genes is rare in patient specimens, we synthesized independent plasmid DNA constructs to express each of the 15 fusion genes (Supplementary Digital Content 3, http://links.lww.com/JTO/A695). Using 0.01 pg of each plasmid construct, the assay detected all targets from both forward and reverse directions, thus validating the specificity and precision of the novel assay. The characteristic mass spectrometric peaks for forward and

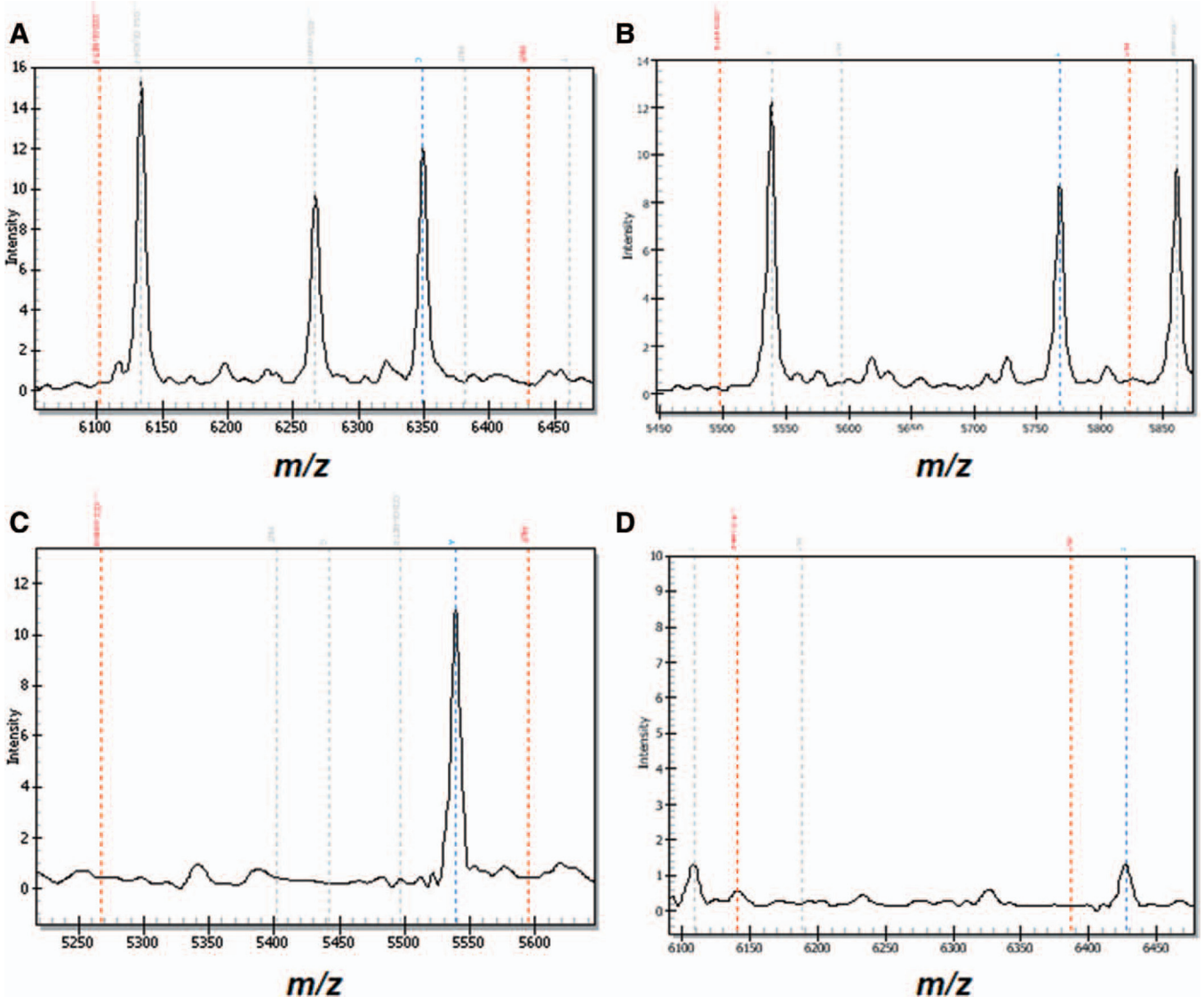



**FIGURE 3.** Detection of CCDC6-RET (C1; R12) fusion gene expression in LC-2/ad cell line complimentary DNA. The characteristic mass spectra are shown for: (*A*) extension primer reaction targeting the fusion junction in the forward direction; (*B*) extension primer targeting the fusion junction in the reverse direction; (*C*) control extension primer targeting the upstream exon junction; (*D*) control extension primer reaction targeting the downstream exon junction.

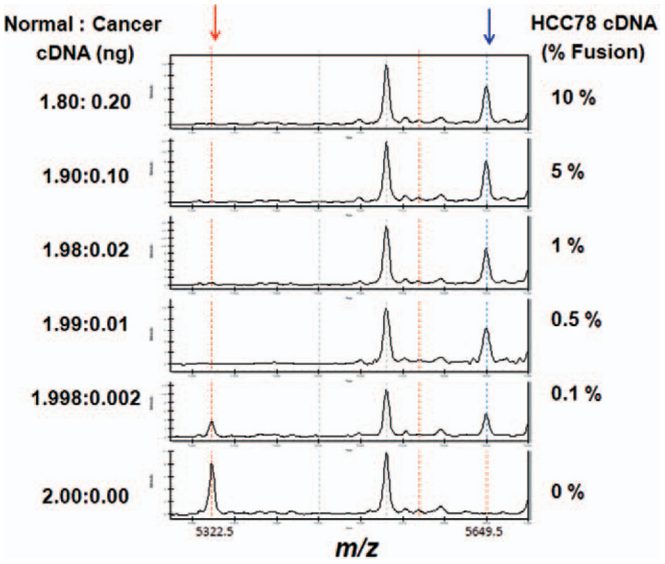



**FIGURE 4.** The sensitivity of the assay was tested by analyzing a series of dilutions of ROS1-SLC34A2 (SL4; R32) fusion gene–positive HCC78 cell line complimentary DNA (cDNA). HCC78 cell line cDNA was spiked into healthy lung cDNA at the indicated ratios to recapitulate a heterogeneous cell population that is presented in patient specimens. The left arrow indicates the predicted mass corresponding to the unextended primer. The right arrow indicates the peak corresponding to the mass of an extension primer reaction accurately detecting the fusion gene junction.

reverse extension primer reactions are given in Supplemental Digital Content 4 (http://links.lww.com/JTO/A696).

The detection of wild-type exon junctions was validated using cDNA derived from lung tissue of healthy individuals. Our multiplexed assay detected all the targeted exon junctions, yielding the predicted mass spectrometric peaks (Supplemental Digital Content 5, http://links.lww.com/JTO/A697). The applicability of the assay was further tested using cDNA from two NSCLC cell lines that are known to express ROS1 and RET fusion genes, HCC78 and LC-2/ad, respectively.[5,15] The ROS1 fusion gene junction comprising ROS1 exon 32 fused to SLC34A2 exon 4 (R32; SL4) along with the upstream and downstream wild-type exon junctions were detected in all the designated wells of the HCC78 cell line cDNA (Fig. 2). The CCDC6-RET (C1; R12) fusion junction together with the upstream and downstream exon junctions was detected in all the designated wells in testing the LC-2/ad cell line cDNA (Fig. 3).

Finally, the sensitivity of the multiplexed assay was tested by analyzing a series of dilutions of HCC78 cell line cDNA so that markedly decreased amounts of the ROS1-SLC34A2 (SL4; R32) fusion gene would be represented in each sample. In contrast to making the dilution series in water or buffer, a concentration of cell line cDNA harboring the fusion gene was spiked into healthy lung cDNA at the ratios indicated in Figure 4. The validation was done in duplicate, and robust accurate mass spectrometry peaks were attained at cancer cell dilutions down to 0.5% (Fig. 4). At a dilution of 0.1% cancer cell cDNA (0.002 ng/well), the corresponding peak was observed in one run, but not the second, thus benchmarking the limits of detection sensitivity at 0.5%.

## DISCUSSION

Here, we have developed the first mass spectrometry–based assay to simultaneously detect the expression of known oncogenic ROS1 and RET fusion genes in NSCLC. The multiplexed approach is well suited for screening scarce tumor material from patient biopsies. In a recent study to detect *ALK* gene rearrangements, RT-PCR outperformed fluorescent in situ hybridization and immunohistochemistry based on sensitivity and specificity[11]; however, the major limitation of RT-PCR is that it requires high-quality RNA, largely limiting its applicability to fresh frozen tissues. Our assay uses the Sequenom MassARRAY system with the capability to use formalin-fixed paraffin-embedded tissues. The novel platform has the capacity to simultaneously test 32 samples. Moreover, the turnaround time of the assay procedure comes in under 8 hours. Testing per sample includes four reactions for each fusion gene target. This includes the detection of the targeted fusion gene sequence in both forward and reverse directions and evaluation of the false-negatives by detection of wild-type exon boundaries upstream and downstream of the fusion point. This is the first multiplexed MassARRAY design that integrates such controls.

Due to the low frequency of ROS1 and RET gene rearrangements in patient specimens, we used synthetic cDNA and biospecimens from NSCLC cell lines to test the specificity and accuracy of each assay in our multiplexed panel. To the best of our knowledge, there are only two cell lines available, derived from NSCLC reported to express ROS1 or RET fusion genes—ROS1-SLC34A2 (SL4; R32) in the HCC78 cell line and CCDC6-RET in the LC-2/ad cell line—and our assay detected both the fusions accurately. With high-throughput sequencing of greater numbers of NSCLC transcriptomes, additional oncogenic variants of the ROS1 and RET fusion genes may be discovered. If it is the case that with further research their identification is verified and oncogenic potential characterized, our MassARRAY test can be readily expanded to include them. In summary, we have provided a robust and flexible screening method that can be integrated with other targeted approaches for low-cost, high-sample throughput analysis.

## ACKNOWLEDGMENTS

*The authors thank Dr. Hema Liyanage for the help with Sequenom MassARRAY software tools and Dr. Michael Hagen for the assistance in running the assays.*

## REFERENCES

1. Ding L, Getz G, Wheeler DA, et al. Somatic mutations affect key pathways in lung adenocarcinoma. *Nature* 2008;455:1069–1075.
2. Seo JS, Ju YS, Lee WC, et al. The transcriptional landscape and mutational profile of lung adenocarcinoma. *Genome Res* 2012;22:2109–2119.
3. Ju YS, Lee WC, Shin JY, et al. A transforming KIF5B and RET gene fusion in lung adenocarcinoma revealed from whole-genome and transcriptome sequencing. *Genome Res* 2012;22:436–445.
4. Kohno T, Ichikawa H, Totoki Y, et al. KIF5B-RET fusions in lung adenocarcinoma. *Nat Med* 2012;18:375–377.

5. Rikova K, Guo A, Zeng Q, et al. Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. *Cell* 2007;131:1190–1203.
6. Takeuchi K, Soda M, Togashi Y, et al. RET, ROS1 and ALK fusions in lung cancer. *Nat Med* 2012;18:378–381.
7. Rimkunas VM, Crosby KE, Li D, et al. Analysis of receptor tyrosine kinase ROS1-positive tumors in non-small cell lung cancer: identification of a FIG-ROS1 fusion. *Clin Cancer Res* 2012;18:4449–4457.
8. Lipson D, Capelletti M, Yelensky R, et al. Identification of new ALK and RET gene fusions from colorectal and lung cancer biopsies. *Nat Med* 2012;18:382–384.
9. Bergethon K, Shaw AT, Ou SH, et al. ROS1 rearrangements define a unique molecular class of lung cancers. *J Clin Oncol* 2012;30:863–870.
10. Drilon A, Wang L, Hasanovic A, et al. Response to Cabozantinib in patients with RET fusion-positive lung adenocarcinomas. *Cancer Discov* 2013;3:630–635.
11. Wu YC, Chang IC, Wang CL, et al. Comparison of IHC, FISH and RT-PCR methods for detection of ALK rearrangements in 312 non-small cell lung cancer patients in Taiwan. *PLoS One* 2013;8:e70839.
12. Sakai K, Okamoto I, Takezawa K, et al. A novel mass spectrometry-based assay for diagnosis of EML4-ALK-positive non-small cell lung cancer. *J Thorac Oncol* 2012;7:913–918.
13. Kudo K, Nishio M, Sakai K, et al. Detection of EML4-ALK in serum RNA from lung cancer patients using MassARRAY platform. *J Clin Oncol* 2012;30.
14. Lambros MB, Wilkerson PM, Natrajan R, et al. High-throughput detection of fusion genes in cancer using the Sequenom MassARRAY platform. *Lab Invest* 2011;91:1491–1501.
15. Matsubara D, Kanai Y, Ishikawa S, et al. Identification of CCDC6-RET fusion in the human lung adenocarcinoma cell line, LC-2/ad. *J Thorac Oncol* 2012;7:1872–1876.